5. Materials and Methods

5.1. DNA constructs

Most constructs used in this work derive from the plasmid pME.A (originally known as pDPL6) made by Haniford and Pulleyblank (Haniford and Pulleyblank 1983) (Figure 5.1). This plasmid is a derivative of pBR322 in which the region between the Hae II sites 778 and 2352 has been deleted and the region between the EcoR I site at position 1 and the Sal I site at position 651 has been replaced by a polylinker. The plasmid pME.A serves as an example of a DNA in the B-DNA conformation since it is devoid of any sequences likely to adopt the Z-DNA conformation. By inserting the sequences $(CG)_{11}$ and $(TG)_{30}$ into the Sma I site, Haniford and Pulleyblank constructed the plasmids pME.E and pME.C (originally named pDHg16 and pDHf14) which are used as examples of plasmids adopting the Z-DNA conformation. The plasmid pME.8 was constructed in a similar way by inserting a $(CG)_8$ sequence into the BamH I site of the polylinker of pME.A. Since pME.A still contains a sequence of six alternating purine and pyrimidine bases, which could adopt the Z-DNA conformation under high degrees of negative supercoiling, this

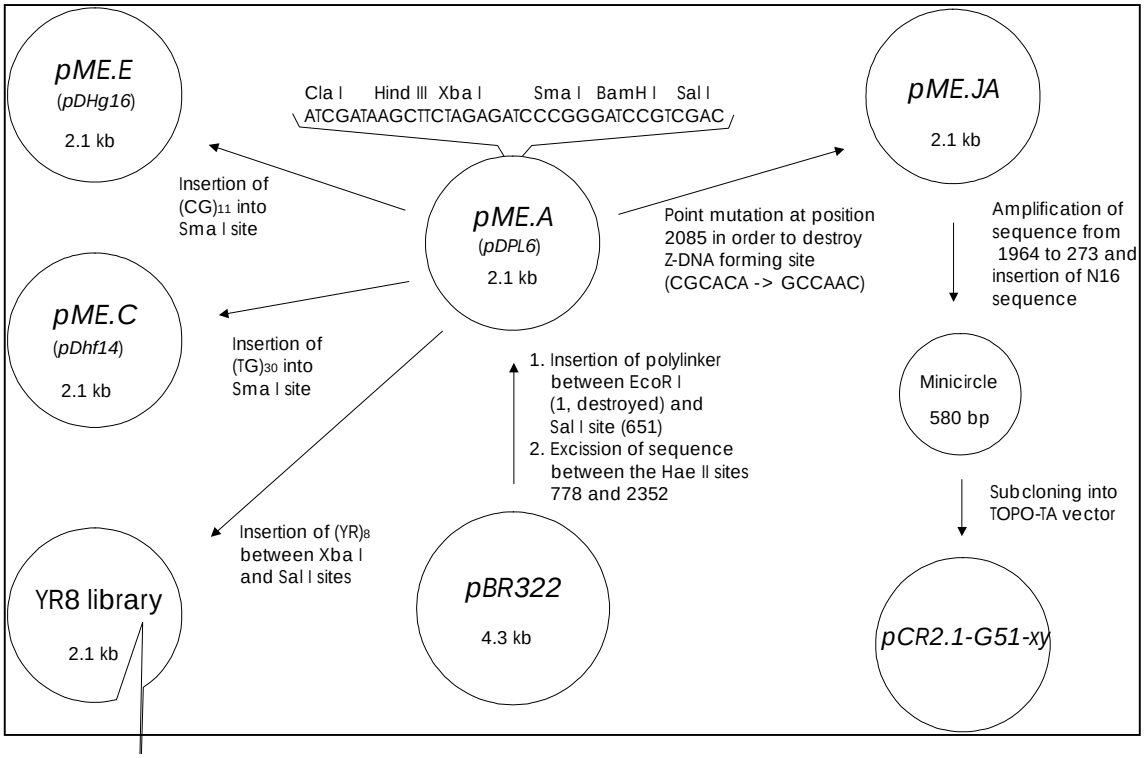

Figure 5.1 DNA constructs

sequence was mutated, yielding plasmid pME.JA. The plasmid pME.A was the template for the generation of the YR8 plasmid library, in which the $(YR)_8$ sequence was inserted between the Xba I and Sal I sites of the polylinker. In contrast, the minicircle libraries were based on the plasmid pME.JA with the sequence from 1943 to 273 used as template for the PCR reaction. The selected minicircles were converted to linear DNA by PCR and inserted into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen).

5.2. Proteins

Z22 antibody — The Z22 antibody is an IgG monoclonal antibody generated from mice immunized with a brominated poly(dG-dC)·poly(dG-dC) polymer and shown to bind to Z-DNA in a sequence independent fashion (Möller et al. 1982). The preparations were a kind gift of Dr. Ky Lowenhaupt and Dr. Stefan Wölfl.

GST-Zα — The plasmid coding for GST-Zα was a kind gift of Dr. Alan Herbert. The vector was constructed by the insertion of the sequence coding for aminoacids 122 to 197 of the ADAR1 protein into the plasmid pGEX-5X1 (Pharmacia). The resultant fusion protein consisted of a N-terminal Glutathione-S-transferase domain (GST) and a C-terminal Zα domain with a Factor Xa cleavage site located between the domains. The total size of the fusion protein was app. 34 kD .

His-Zα — The plasmid coding for His-Zα, also known as Z-long or His-Zα76v, was a kind gift of Dr. Alan Herbert and was constructed by excising the coding sequence from the plasmid pGEX-5X1 and inserting it into the plasmid pET28a (Novagen). The sequence of the fusion protein is as follows :

```
ATG GCC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG
M   G   S   S   H   H   H   H   H   H   S   S   G   L   V

        ⇓
CCG CGC GGC AGC CAT ATG GGA GGT GTT GAT TGC CTT TCC TCA CAT
P   R   G   S   H   M   G   G   V   D   C   L   S   S   H

TTC CAG GAA CTG AGT ATC TAC CAA GAT CAG GAA CAA AGG ATC TTA
F   Q   E   L   S   I   Y   Q   D   Q   E   Q   R   I   L

AAG TTC CTG GAA GAG CTT GGG GAA GGG AAG GCC ACC ACA GCA CAT
K   F   L   E   E   L   G   E   G   K   A   T   T   A   H

GAT CTG TCT GGG AAA CTT GGG ACT CCG AAG AAA GAA ATC AAT CGA
D   L   S   G   K   L   G   T   P   K   K   E   I   N   R

GTT TTA TAC TCC CTG GCA AAG AAG GGC AAG CTA CAG AAA GAG GCA
V   L   Y   S   L   A   K   K   G   K   L   Q   K   E   A

GGA ACA CCC CCT TTG TGG AAA ATC GAA TTC CCG GGT CGA CTC GAG
G   T   P   P   L   W   K   I   E   F   P   G   R   L   E

CGG CTG ACT GAC TGA
R   L   T   D   *
```

The Thrombin cleavage site is indicated by an arrow. The aminoacids belonging to the ADAR1 protein are underlined. The five aminoacids following the cleavage site do not belong to the ADAR1 protein, but are encoded in the plasmid vector. Likewise the C-terminal sequence EFPGRLERLTD is encoded by the vector. The total number of aminoacids of the fusion protein is 110, resulting in a molecular weight of 12,353 Da.

For several experiments the Zα peptide was used after the affinity tag had been cleaved off. In the case of circular dichroism spectroscopy GST-Zα was used as source, in the case of several of the band shift reactions the His-Zα peptide was the source. In these cases the protein preparations were purified after proteolysis using anion exchange FPLC and were a kind gift of Dr. I. Berger and Mr. T. Schwartz.

5.3. Electrophoretic separation of DNA in agarose gels

Agarose gels were used for the gel electrophoretic separation of double stranded DNA molecules with sizes between 100 bp and 10,000 bp. Depending on the sizes of the DNA molecules different concentrations of agarose gels were used (Table 5.1). TAE buffer was used and in most cases ethidium bromide was added from a 0.5 mg/ml stock solution to a final concentration of 0.1 µg/ml. Samples were mixed with 1/10 volume of loading buffer (FicPuStop) and loaded onto the gel. Following electrophoresis the gel was exposed to ultraviolett light of 254 nm wavelength. If the DNA was to be recovered from the gel a small aliquot of the DNA was loaded on a lane next to a marker DNA. After electrophoresis these two lanes were cut from the gel and exposed to ultraviolett light. The position of the desired DNA band was marked and the markings used to cut the DNA band from gel.

(%) Gel (w/v)	Length of the linear DNA
0.6	1,000 - 20,000
0.7	800 - 10,000
0.9	500 - 7,000
1.2	400 - 6,000
1.5	200 - 4,000
2.0	100 - 3,000

Table 5.1 : Agarose concentration used for optimal resolution

5.4. Electrophoretic separation of DNA in acrylamide gels

5.4.1. Gel electrophoresis of DNA minicircles

The size of the DNA minicircles did not allow for good resolution of topoisomers in agarose gels. Therefore, DNA minicircles were usually electrophoresed on 4 % (w/v) native acrylamide gels. A gel solution of 20 ml volume containing 17 ml deionized water, 1 ml 10 x TBE buffer and 2 ml 40 % (w/v) acrylamide / bisacrylamide (29:1) solution was prepared and the polymerization reaction started by the addition of 200 µl of a 25 % (w/v) APS solution and 20 µl TEMED. The gel was pre-electrophoresed for one hour at

100 V and the samples applied and electrophoresed at 100 V until the Bromphenol Blue dye left the gel. Due to the low amounts of minicircle DNA available for analysis, the gel was stained with the Sybr-green dye (Molecular probes). A few drops of a 1:10,000 dilution of the dye stock solution were placed directly on an UV-transilluminator and the gel placed on top. After a few minutes the illuminator was turned on and the gel exposed to UV light of 254 nm wavelength. While the sensitivity of the Sybr-green dye is very high, the quality of the pictures obtained is lower than that usually obtained with ethidium bromide staining, since often spurious background signals are observed.

5.4.2. Gel purification of DNA oligonucleotides

Depending on the use of the oligonucleotides, two different procedures for gel purification were used. In the case of long oligonucleotides (> 60 nucleotides) used as PCR primer, a denaturing acrylamide gel of appropriate concentration was prepared using a comb with wide slots. After electrophoresis the DNA band of interest was located by UV-shadowing : The gel was placed on a TLC plate containing a fluorescent chromophore and exposed to UV light of 254 nm wavelength. Under these conditions the DNA band showed as a dark band since it absorbs the ultraviolett light. The gel piece was excised, placed in a microcentrifuge tube and crushed. An appropriate volume of buffer was added and the tube allowed to stand overnight. The next day the gel pieces were pelleted by centrifugation in an Eppendorf centrifuge at 13,000 rpm and room temperature for 5 minutes and the supernatant recovered. In the case of radioactively end labeled oligonucleotides a native polyacrylamide gel of between 15 % and 20 % concentration (w/v), 1 mm thickness and 1 cm wide slots was used. After electrophoresis the gel was wrapped in Saran-Wrap and phosphorescent markers attached. A x-ray film was exposed to the gel for 5 minutes and developed. Using the image left by the markers, the film was aligned on the gel and the DNA bands of interest excised. The gel pieces were placed in the upper chamber of a Spin-X 100 device (Costar) and carefully crushed using a pipette tip. To the crushed gel 300 µl of a solution containing 50 mM NaCl and 10 mM Tris/HCl pH 8.0 was added and incubated overnight at room temperature. The next day the Spin-X 100 device was centrifuged in an Eppendorf microcentrifuge at 15,000 g and room temperature for 5 minutes. The upper

chamber containing the crushed gel pieces was discarded and the aqueous solution in the lower tube containing the eluted DNA used for further experiments.

5.4.3. Detection of DNA in acrylamide gels by silver staining

Silver staining was used to detect low amounts of DNA in polyacrylamide gels. During silver staining it proved important to wear gloves when handling the gel, since fingerprints on the gel will be stained too. Following electrophoresis the gel was washed twice for 3 minutes in a solution of 0.5 % (v/v) acetic acid in 10 % (v/v) ethanol. The gel was then incubated for 10 minutes in 100 ml of a 0.1 % (w/v) $AgNO_3$ solution. After a quick rinse in deionized water the gel was stained for approximately 20 minutes in 100 ml of a freshly prepared solution of 1.5 % (w/v) NaOH, 0.01 % (w/v) $NaBH_4$ and 0.4 ml 37 % (v/v) formaldehyde. Once a noticeable background coloration developed the staining reaction was stopped by a 10 minute incubation in 100 ml of a 0.75 % (w/v) solution of Na_2CO_3.

5.5. Bandshift assays

A typical sample of a bandshift assay contained 10,000 cpm of radioactively labeled and gel purified DNA probe and 0.5 – 1.0 µg recombinant protein. The binding buffer consisted of 5 mM $MgCl_2$ and 0.5 x TB. The samples were incubated for 1 h at room temperature. Prior to electrophoresis one tenth volume of a 25 % (w/v) solution of Ficoll 4000 was added to increase the density of the sample. Usually no Bromphenol Blue was added. The samples were resolved on 5 % (w/v) 29:1 non-denaturing acrylamide gels. In order to obtain sharp bands, gels of 1 mm thickness and 20 cm length and slots of 1 cm width were used. The gels were mounted in a Hoefer SE500 gel electrophoresis apparatus in such a way that a reservoir containing 4 l buffer surrounded and cooled the gel. The electrophoresis buffer consisted of 0.5 x TB. Following pre-electrophoresis at 100 V for 1 h the samples were loaded and resolved at 500 V for 15 minutes. Following electrophoresis, the gels were lifted onto Schleicher & Schüll GB002 blotting paper, covered with Saran-Wrap, and dried for 20 minutes at 80 ºC under vacuum. For detection, a Phosphorimager cassette was exposed to the gel over night.

5.6. Circular dichroism spectroscopy

Measurements were performed on an Aviv Model 62 DS CD-spectrometer. Methylated DNA polymer poly(m^5CG)·poly(m^5CG) (Pharmacia, Piscataway, NJ, average length 2,900 basepairs) was dissolved in deionized water and used without further purification. All measurements were done in a temperature controlled quarz cuvette with a pathlength of 1 cm at a temperature of 30 ºC in a buffer of 25 mM NaCl, 0.1 mM Na_2·EDTA and 50 mM Tris/HCl (pH 7.4) and a DNA concentration of 46 µM (basepairs). After addition of peptide, the samples were placed in the spectrometer and equilibrated for 15 minutes prior to data acquisition. Spectra were collected in 1 nm teps using a slit width of 1.5 nm and a measuring time of 2 seconds. For analysis, the measured curves were smoothed using the smooth.com program supplied by Aviv, and the circular dichroism spectrum of the buffer subtracted.

5.7. Electroporation

5.7.1. Preparation of competent bacteria by $CaCl_2$ treatment

Chemically competent bacteria were prepared using the $CaCl_2$ method. A 10 ml culture of *E. coli* was grown overnight in LB medium at 37 ºC and used to inoculate a 400 ml culture. The bacteria were grown for 4 to 6 h until an OD_{550} of between 0.4 and 0.5 was reached. The culture was chilled on ice and pelleted by centrifugation in a Sorvall GSA rotor at 5,000 rpm (4,000 g) and 4 ºC for 10 minutes. The pellet was resuspended in 200 ml of ice cold sterile 0.1 M $CaCl_2$. The cells were incubated on ice for 30 minutes with occasional stirring and then pelleted as before. The cells were resuspended in 20 ml ice cold sterile 0.1 M $CaCl_2$ and 15 % (w/v) glycerol. Aliquots were pipetted in autoclaved microcentrifuge tubes and stored at –70 ºC. Aliquots were not refrozen after thawing, but discarded.

5.7.2. Preparation of competent bacteria for electroporation

Prior to the transformation of bacteria by the electroporation method, the bacteria have to be resuspended at high density in a solution of low electrical conductivity. A single bacterial colony on an agar plate was used to inoculate a 10 ml culture in LB medium and shaken overnight at 37 ºC. The next day 8 ml of the overnight culture were used to inocculate a 800 ml culture which was shaken vigorously at 37 ºC until the optical density reached between 0.5 and 0.6 OD_{600} . The bacteria were then placed on ice for 30 minutes. From now on the bacteria were always stored and handled on ice. The bacterial suspension was transferred into 4 precooled and autoclaved centrifuge buckets and pelleted in a precooled Sorvall GSA rotor at 5,000 rpm (4,000 g) at 4 ºC for 5 minutes. The supernatant was discarded and the bacterial pellet carefully resuspended on ice in 50 ml precooled autoclaved Milli-Q water. Milli-Q water was then added to a total of 200 ml and the bacteria pelleted by centrifugation as before. The supernatant was discarded and the bacteria again resuspended in Milli-Q water, except that the contents of two buckets each were combined. After another centrifugation the bacteria were resuspended in 16 ml of a 10 % (w/v) solution of glycerol in Milli-Q water per bucket and the bacteria resuspended. The bacterial suspensions were pooled in a 50 ml Falcon tube and pelleted by a final centrifugation. The supernatant was discarded and the bacteria resuspended either in the remaining liquid, or 1.6 ml of a 10 % (w/v) solution of glycerol were added. For long term storage the bacterial suspension was aliquoted in 1.5 ml microcentrifuge tubes and stored at –70 ºC.

5.7.3. Electroporation of bacteria

For electroporation of bacteria a Gene-Pulser apparatus manufactured by BioRad was used together with electroporation cuvettes of 0.2 mm thickness. The settings were : 400 Ω and 2.5 µFd. The bacterial suspension was thawed on ice and between 40 µl and 80 µl of the bacterial suspension mixed with between 1 µl and 2 µl of DNA solution. Since, for successful electroporation, the suspension has to have a low electrical conductivity, the DNA solution had been desalted either by precipitation with ethanol or by passing the solution through a size exclusion spin column (Pharmacia G-25). The mixture of

DNA and bacterial suspension was transferred into a precooled electroporation cuvette, placed into the apparatus mentioned above and electroporated. Immediately afterwards, the bacteria were resuspended in 1 ml of SOC medium, transferred to an Eppendorf tube and incubated for 1 h at 37 ºC on a shaking platform. Between 10 µl and 200 µl of bacterial suspension was plated onto LB agar plates (100 mm ∅) depending on the expected number of colonies. Colonies were usually visible after a 16 h incubation at 37 ºC.

5.8. Preparation of DNA

5.8.1. Small scale preparation of plasmid DNA

For small scale preparation of plasmid DNA a 5 ml culture of LB medium containing the appropriate antibiotic was inoculated with a single bacterial colonies using a toothpick. The culture was placed at 37 ºC in a shaking incubator for 8 to 36 hours. The preparation of the plasmid DNA was accomplished using the Miniprep Kit from Qiagen. The buffers P1, P2, N3, PE and EB were supplied by the manufacturer. First 1.5 ml of the bacterial suspension (3 ml in the case of low copy number plasmids) were transferred to microcentrifuge tubes and the bacteria pelleted by a short centrifugation in an Eppendorf microcentrifuge at 10,000 rpm and room temperature for 20 seconds. The supernatant was discarded and the bacteria resuspended in 250 µl buffer P1 by vortexing. The bacteria were lysed for 4 minutes by addition of 250 µl buffer P2 and chromosomal DNA and cellular debris precipitated by addition of 350 µl buffer N3. Following centrifugation in an Eppendorf microcentrifuge at 13,000 rpm and room temperature for 10 minutes the supernatant was applied to spin columns. The columns were centrifuged in an Eppendorf microcentrifuge at 13,000 rpm and room temperature for 1 minute and 750 µl buffer PE applied. The columns were centrifuged as before and the flowthrough discarded. The spin columns centrifuged as before for another minute. The spin columns were placed into Eppendorf tubes and 50 µl of elution buffer EB applied. After one minute the spin columns were centrifuged as before in order to collect the eluate.

5.8.2. Large scale preparation of plasmid DNA

Large scale preparations of plasmid DNA were done using the Maxiprep kit from Qiagen. The buffers P1, P2, P3, QBT, QC and QF were supplied with the kit. Between 200 and 300 ml of LB medium containing the appropriate antibiotic were inoculated with 1 ml of a saturated bacterial culture. The cultures were grown at 37 ºC until saturation. However, if very high quality supercoiled DNA was required, the bacteria were harvested when the cultures reached an OD_{600} of 1,3 – 1,5 . The bacterial cultures were transferred to 200 ml centrifuge bottles and centrifuged in a GSA rotor at 4,000 rpm (2,600 g) and 4 ºC for 15 minutes. The supernatant was discarded and the bacterial pellet resuspended on ice in 10 ml buffer P1. In order to achieve lysis, 10 ml of buffer P2 were added and carefully mixed by turning the tube. After four minutes 10 ml of buffer P3 were added, carefully mixed and allowed to stand for 20 minutes on ice. The precipitate was pelleted by centrifugation in a GSA rotor at 10,000 rpm and 4 ºC for 30 minutes. If the supernatant was still cloudy, it was transferred to a new centrifuge bottle and centrifuged again. The cleared supernatant was applied to an anion exchange column equilibrated with 10 ml buffer QBT. The column was washed with 60 ml buffer QC and the plasmid DNA eluted with 15 ml buffer QF. The eluted DNA was precipitated with 10.5 ml isopropanol and followed by centrifugation in a SS-34 rotor at 10,000 rpm (12,000 g) and 4 ºC for 30 minutes. The DNA pellet was rinsed with 70 % (v/v) ethanol and dried. The DNA was usually dissolved in TE pH 8.5 and stored at –20 ºC.

5.8.3. Precipitation of DNA with ethanol

To the DNA solution 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of 96 % (v/v) ethanol pre-cooled to –20 ºC were added and the sample mixed by vortexing. If the initial DNA concentration was higher than 100 ng/ml the sample was centrifuged in an Eppendorf microcentrifuge at 13,000 rpm and 4 ºC for 30 minutes. The supernatant was discarded, 200 µl of icecold 70 % (v/v) ethanol added, mixed by vortexing and centrifuged as before for 5 minutes. The supernatant was discarded and the DNA pellet allowed to dry at room temperature. To speed up the drying process the sample was given a short spin in an Eppendorf microcentrifuge and any residual liquid carefully

pipetted off. If the initial concentration of DNA was low, or the DNA was smaller than 100 bp, 1 µl of a 5 mg/ml solution of linear polyacrylamide (Ambion) was added as carrier. In addition an incubation of at least 30 minutes at –70 ºC was included before the centrifugation.

5.8.4. UV-spectrophotometric determination of nucleic acid concentrations

The nucleic acid solution of interest was diluted to a suitable concentration (for example for a DNA concentration of 1 µg/µl a 1:100 dilution was useful) in a final volume of 300 µl. A quartz cuvette was filled with 300 µl dilution buffer and the reading of the photometer adjusted to zero. The buffer was discarded and replaced by the diluted nucleic acid solution. The absorption spectrum of the solution was measured and the extinction at a wavelength of 260 nm used to calculate the concentration of the nucleic acid. The following equations were used :

double-stranded DNA	1 OD260 equals 50 µg
single-stranded RNA	1 OD260 equals 40 µg
single-stranded DNA	1 OD260 equals 30 µg

5.8.5. Determination of DNA concentration using the fluorescent dye Hoechst 33258

In order to determine the concentration of samples containing only low amounts of DNA the fluorescent dye Hoechst 33258 was used in conjunction with the fluorometer TK100 (Hoefer). A working dye solution was prepared by combining 45 ml Milli-Q water, 5 ml of 10 x TEN and 5 µl of a 10 mg/ml solution of the Hoechst 33258 dye. Since the binding of the dye to DNA is not totally independent of the DNA sequence, all measurements using this system are measurements relative to a DNA standard. The plasmid pME.JA was chosen as standard, since it most closely resembled the nucleic acid libraries used for *in vitro* selection. Prior to the measurements, the photometer was adjusted by adding 2 ml of the working dye to the measuring cuvette and, with the scale knob turned maximally to the left, the reading adjusted to zero with the gain knob. Then either 1 or 2 µl of a 100 ng/µl solution of the plasmid pME.JA was added, the cuvette covered with Parafilm and

mixed. The cuvette was placed back in the photometer and the reading adjusted to 50 or 100 using the scale knob. For measurements the cuvette was rinsed thoroughly with deionized water and washed once with 1 ml of dye solution. Then 1 or 2 µl of DNA solution were added to 2 ml of working dye solution and measured in the photometer.

While this method allowed the measurement of dilute DNA solutions, the accuracy of the measurements appeared to be not very high. However, it proved sufficinedt to measure DNA concentrations down to 10 ng/µl. It was observed that the photometer readings were dependent on the amount of stray light reaching the measurement chamber from the laboratory. It therefore proved useful to place the photometer away from direct sunlight in a room illuminated with artificial light. The working dye proved to have a limited shelf life after which the measurements showed large fluctuations and therefore was made freshly every week.

5.9. DNA sequencing

DNA sequencing was performed using the Thermo Sequenase kit with 7-deaza-dGTP and a LiCor 4000L sequencer. The underlying principle is the use of oligonucleotide primers containing an infrared label at their 5´ end. In a primer extension reaction a thermostable DNA polymerase and dideoxynucleotides were used to produce DNA ladders for the individual bases. Due to the use of a thermostable enzyme the reaction can be cycled, resulting in increased sensitivity. In a standard protocol each reaction was prepared by combining in a well of a 384 well plate 2 µl of the respective A-, C-, G- or T-reagent, 1 µl of miniprep DNA and 2 pmol primer. The plate was covered with a Microseal™A film (MJ Research) and placed in a thermocycler. The following temperature program was used : 5´ 95 ºC, (30´´ 95 ºC, 30´´ 55 ºC, 30´´ 72 ºC) x 40. The reactions were stopped with a Formamide/dye mix and a small volume (0.5 µl – 1.0 µl) applied to 6 % (w/v) PAGE gels of 0.2 mm thickness. Electrophoresis and detection were accomplished using the automated system of the sequencer.

In the case of lowcopy plasmids the DNA concentration of the small scale plasmid preparation is too low to achieve acceptable results using the standard protocol. In these cases a modified protocol was used, which has been termed the ´semi-exponential

sequencing method´ (Sarkar and Bolander 1995). This method includes a second primer in the reaction, chosen in such a way that it hybridizes, compared to the sequencing primer, to the opposite strand of the DNA at a position on the far site of the sequence of interest. Hence, more sequencing template is generated during the temperature cycles of the sequencing reaction. A sequencing reaction was prepared by combining 1 pmol labeled primer, 0.5 pmol reverse primer, 25 ng plasmid DNA and 2 µl of the respective A-, C-, G- or T-reagent in a total volume of 8 µl. The temperature program contained an initial 5´ incubation at 95 ºC followed by 30 cycles of 30 seconds 95 ºC, 30 seconds 45 ºC and 30 seconds 72 ºC.

5.10. DEPC reactions

For DEPC modification 1.5 µg plasmid DNA (equivalent to 0.5 pmol) was incubated in 100 µl buffer 1 (50 mM Na-Cacodylate pH 7.1, 1 mM EDTA) on ice. 3 µl DEPC (Sigma) were added, the sample vortexed vigorously and incubated for the specified time at room temperature. The reaction was stopped by addition of 3 µg tRNA (Sigma), and precipated by addition of 10 µl ice cold 3 M sodium acetate pH 5.2 and 250 µl ice cold ethanol. After centrifugation the pellets were resuspended in 100 µl Milli-Q water and precipitated again. The DNA samples were linearized with the endonuclase Sma I and precipitated. The linearized DNA was resuspended in 10 µl Milli-Q water, transferred to 200 µl reaction tubes and incubated for 30 minutes at 90 ºC . Primer extension reactions were prepared by combining 0.8 mM $MgCl_2$, 0.25 mM dNTP´s, 0.5 pmol primer M13rever (IR-CAGGAAACAGCTATGACCATG), 2 µl heat treated DNA and 2.5 U Taq DNA polymerase in a total volume of 10 µl . A thermocycler protocol of 5´ 95 ºC followed by 40 cycles of 30´´ each at 95 ºC, 55 ºC and 72 ºC was used and the samples resolved on a LiCor 4000L sequencer.

5.11. Enzymatic reactions

5.11.1. Radioactive end labeling of DNA oligonucleotides

For radioactive end labeling of oligodeoxynucleotides with T4 Polynucleotidekinase 2.5 µl of 10 x buffer supplied with the enzyme, 10 pmol of oligonucleotide and between 10 and 100 µCi of $\gamma[^{32}P]$-ATP (6,000 mCi/mmol) were combined and Milli-Q water added to a final volume of 24 µl. The reaction was started by addition of 1 µl (10 U) of enzyme. The sample was placed at 37 ºC for 1 hour followed by a 20 minute inactivation of the enzyme at 65 ºC. In the case of hairpin oligonucleotides the sample was heated to 90 ºC in a waterbath, after which the waterbath was turned off and allowed to slowly cool to room temperature.

5.11.2. Restriction of DNA

DNA was treated with restriction enzymes according to the manufacturers instructions using incubation times between 1 hour and 18 hours, avoiding addition of more than 1/10 volume of enzyme solution to prevent star activity due to excessive glycerol concentration.

5.11.3. Dephosphorylation of DNA

DNA was dephosphorylated using either Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim) or Shrimp Alkaline Phosphatase (Amersham Life Science). In the case of Calf Intestinal Alkaline Phosphatase inactivation of the enzyme was achieved by addition of EDTA to 10 mM, a 20 minute incubation at 70 ºC and two Phenol/Chloroform extractions. In the case of the Snail Alkaline Phosphatase inactivation was achieved by heating the sample to 65 ºC for 20 minutes.

5.11.4. Ligation of DNA

Ligations used to insert DNA into plasmids were prepared by combining vector DNA and insert DNA together with 1 U T4 DNA Ligase and the buffer supplied by the

manufacturer in a final volume of 10 µl. The ligation samples were incubated overnight at 16 ºC.

5.12. Site directed mutagenesis

Milli-Q water	35 µ
10 x buffer	5 µl
2 mM dNTP´s	5 µl
50 mM $MgCl_2$	1 µl
50 µM Bio-3´pMEAB	1 µl
50 µM mpME.JA	1 µl
pME.A 0.1 mg/ml	1 µl
pfu DNA Polymerase 7.5 U/µl	1 µl

Table 5.2

The mega-primer protocol (O.Landt et al. 1990) was used for site directed mutagenesis. In a first PCR reaction a megaprimer was produced using the primers Bio-3´pMEAB (TTG CCG CCG CAA GGG CAT CGG TCG) and mpME.JA (AGG GGT TCC GGC CAA CTT TCC CCG) and the plasmid pME.A as template (Table 5.2). The plasmid DNA had been previously linearized with Pst I. In order to prevent the introduction of undesired mutations, the high fidelity DNA Polymerase pfu was used. The temperature program consisted of 10 cycles of 95 °C and 60 °C each. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen) and used together with the primer pMEA.PvuI (CGG TCC TCC GAT CGT TGT C) as megaprimer in a second PCR reaction (Table 5.3). The temperature program consisted of 15 cycles of 95 °C, 56 °C and 72 °C for 1 minute each. The PCR product was cut with Pvu I and BamH I and inserted into plasmid pME.A cut with Pvu I and BamH I. The ligation mix was transformed into *E. coli* bacteria, single colonies were picked and the success of the mutation confirmed by sequencing.

Milli-Q water	29 µl
10 x buffer	5 µl
2 mM dNTP´s	5 µl
50 mM $MgCl_2$	1 µl
50 µM pMEA.PvuI	1 µl
Megaprimer	10 µl
pfu DNA Polymerase 7.5 U/µl	1 µl

Table 5.3

5.13. Protein methods

5.13.1 Gel electrophoresis of proteins

Instead of the more common Laemmli system, the system of Schaegger and Jagow was used for the electrophoresis of proteins, since it provides better resolution of small proteins and peptides. This type of gel is also known as ´Tricine Gel´. ´Tricine Gel´s consists of three layers : resolving gel, spacer gel and stacking gel. In a minigel the height of the resolving gel should be 4 cm, the spacer gel should be 1 cm high and the stacking gel 1 cm high. Recipes for the gel solutions are given in Table 5.2. The resolving and spacer gels were prepared at the same time and poured immediately afterwards. The spacer gel was layered carefully on the stacking gel. Afterwards 2-butanol was layered on top and the gel solutions allowed to polymerize. After the 2-butanol had been discarded the stacking gel was poured.

	Resolving gel	**Spacer gel**	**Stacking gel**
deionized water	1.25 ml	2.4 ml	8.15 ml
40 % (w/v) Acrylamide / N,N´-Methylenebis-acrylamide 29:1	6.25 ml	1.6 ml	1.25 ml
Gel buffer	5.0 ml	2.0 ml	3.1 ml
80 % (w/v) Glycerol	2.5 ml	-	-
APS 25 % (w/v)	100 µl	50 µl	100 µl
TEMED	10 µl	5 µl	10 µl

Table 5.2

Gel buffer	3 M Tris Base, 0.3 % SDS (w/v), pH 8.45
Anode buffer	0.2 M Tris Base, pH 8.9
Kathode buffer	0.1 M Tris Base, 0.1 M Tricine, 0.1 % SDS (w/v), pH 8.25

5.13.2. Purification of recombinant proteins from *E. coli*

5.13.2.1. Purification of the GST-Zα fusion protein

For the preparation of GST-Zα fusion protein, bacteria from a frozen stock were streaked on a LB agar plate containing Ampicillin. The next day a 10 ml overnight culture was inoculated with a single colony and placed overnight in a shaking incubator at 37 ºC. The next day 4 ml each of the saturated overnight culture were used to inoculate

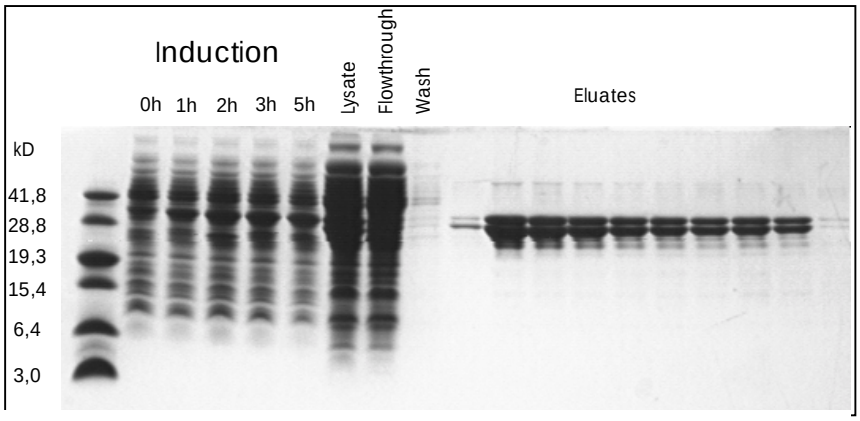

Figure 5.2 Purification of GST-Zα

two 400 ml cultures. After the cultures reached an OD_{600} of 1.0 the synthesis of the fusion protein was induced by addition of IPTG up to a final concentration of 1 mM. The cultures were incubated for another 3 – 5 hours in a shaking incubator after which the cultures were transferred to 200 ml centrifuge bottles and the bacteria recovered by a centrifugation in a GSA rotor at 4,500 rpm at 4 ºC for 20 minutes. The supernatant was discarded and the bacterial pellets stored at –20 ºC or -70 ºC until further processing.

The fusion protein was purified by a one step affinity chromatography. Before starting all buffers were placed on ice. The actual chromatography was performed in a 4 ºC cabinet. The bacterial pellets were thawed on ice, resuspended in 10 ml buffer A (50 ml PBS; 10 µl 0.5 M EDTA; 5 µl ß-Mercaptoethanol; 50 µl PMSF 17 mg/ml; 0.1 % (w/v) Triton X-100) and transferred to a SepCor centrifuge tube. The bacteria were lysed by sonification with a microtip using 10 cycles of sonification and incubation on ice for 30 s each. The lysate was cleared of unlysed bacteria and cellular debris by centrifugation in a SS-34 rotor at 15,000 rpm and 4 ºC for 15 minutes. Recombinant protein was adsorbed to the affinity chromatography resin either in a batch process or by using a premade column. In either case the matrix was prepared by washing 2 ml of GSH-Sepharose (Pharmacia) between 3 and 4 times in 10 ml buffer A. The equilibrated resin was either added to the bacterial lysate, rocked for 1 hour on ice, and poured into a plastic column (batch procedure), or poured immediately into a plastic column and the bacterial lysate loaded onto the packed column (column procedure). The column was washed with 10 – 20 bed volumes of buffer A and the protein eluted with 10 ml elution buffer (150 mM NaCl, 0.25 mM EDTA, 60 mM Tris/HCl pH 8.0; 10 mM reduced Glutathione; 0.2 mM PMSF; 2.5 µl ß-Mercaptoethanol). Aliquots of 1.5 ml volume were collected and stored at 4 ºC. The fractions were analyzed on an acrylamide gel and those fractions containing the desired protein were pooled, transferred to dialysis tubing

(SpectraPor CE MWCO 2,000) and dialyzed overnight at 4 ºC against 2 times 2 l PBS. Protein samples were stored at 4 ºC. A Tricine gel of a typical purification is shown in Figure 5.2 .The protein recovered from the column was not absolutely pure and showed two major bands. For purposes of gel shift analyses and *in vitro* selection these protein preparations proved to be sufficient.

5.13.2.2. Purification of the His-Zα fusion protein

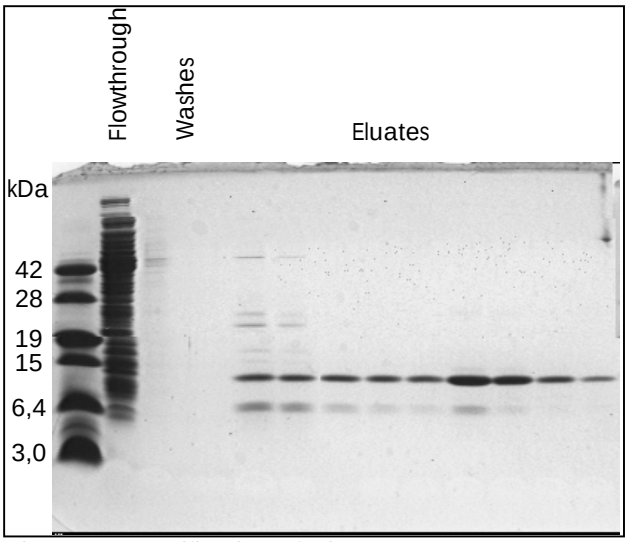

Figure 5.3 Purification of His-Zα

The His-Zα fusion protein was purified on either of two different affinity resins. The underlying principle of both resins is the fact that stretches of consecutive Histidine amino acids will bind to matrixes in which a polyvalent metal ion such as Ni^{2+}, Co^{2+} or Zn^{2+} is chelated in such a way as to leave two coordination sites for binding by Histidine. The Ni-NTA resin (Qiagen) uses Ni^{2+} whereas the Talon resin (Clontech) utilizes Co^{2+} . Growth and lysis of the bacteria was done according to the protocol for the GST-Zα fusion protein except that the bacterial pellets were resuspended in the appropriate binding buffer. In the case of the Talon resin the bacterial pellet was resuspended in 20 ml sonication buffer (100 mM NaCl; 20 mM Tris/HCl pH 8.0) and Lysozyme added to a final concentration of 0.75 mg/ml. Following a 20 minute incubation at room temperature the bacteria were lysed by sonification. The Talon resin was added and the fusion protein allowed to bind during a 20 minute incubation at room temperature. The resin was washed twice in sonification buffer by centrifugation in a clinical centrifuge at 700 g at 4 ºC for 5 minutes. The resin was loaded in a plastic column, washed with 40 ml of wash buffer (2 M NaCl; 50 mM Tris/HCl pH 8.0) and the protein eluted in 10 ml of elution buffer (100 mM NaCl; 50 mM Imidazole; 20 mM Tris/HCl pH 8.0;). The result of a typical purification is shown in Figure 5.3 . The predominant protein has an apparent molecular weight in close agreement with the predicted size of His-Zα (12.4 kD).

5.13.3. Determination of the protein concentration

For determination of protein concentrations the Micro BCA Protein Assay Reagent Kit from Pierce (cat. no. 23235) was used. A set of 10 protein standards was prepared by serially diluting a stock solution of BSA (2 mg/ml) in steps of 1:2 (30 µl volume). If possible, the same buffer was used for the dilution as had been used to prepare the protein of interest. A 96 well microplate was labeled and 10 µl of protein standard pipetted into individual wells. Likewise different volumes of protein solution were pipetted into wells. Buffer was added to a final volume of 100 µl. Several wells were filled with 100 buffer to serve as blanks. The working reagent solution was prepared by combining 50 parts of Reagent MA, 48 parts of Reagent MB and 2 parts of Reagent MC. The working reagent was thoroughly mixed by vortexing. To each well 100 µl of working reagent were added and the microplate covered with adhesive tape. The plate was then incubated for 2 hours at 37 ºC, after which the absorbance was read at 570 nm using a Molecular Devices Emax microplate reader.

5.14. *In vitro* selection of plasmids

5.14.1. Preparation of the plasmid library

Milli-Q water	755 µl
10 x buffer	100 µl
Bio-XbaI primer 1 µg/µl	5 µl
Bio-BamHI primer 1 µg/µl	5 µl
YR8SalI oligonucleotide 1 µg/µl	1 µl
50 mM $MgCl_2$	30 µl
2 mM dNTP´s	100 µl
Taq DNA Polymerase 5 U/µl	5 µl

Table 5.5

The strategy for the generation of the plasmid library was adapted from a procedure developed for phage display selection (Smith 1992). In short, a chemically synthesized DNA oligonucleotide was amplified and double-stranded using biotinylated PCR primers containing restriction sites. After digestion with a restriction endonuclease, the cleaved of ends were removed by adsorption on Streptavidin Agarose beads. The purified fragment containing the randomized nucleotides was ligated into a linearized plasmid vector.

The oligonucleotide YR8SalI (AGA CTC GAT ACG GGA TTA GCT CGA GYR YRY RYR YRY RYR YRC TCG AGC AGG CTT GAA TCT GCT AAG GA) was double-stranded and amplified in a PCR reaction using the primers Bio-XbaI (GAG CTC TAG ACT CGA TAC GGG ATT AGC T) and Bio-BamHI (ACC GCG GAT CCT TAG CAG ATT CAA GCC TG). The PCR reaction was prepared as listed in Table 5.5 , split into 100 µl aliquots, and a program of 5 cycles of 1´ 95 ºC, 1´ 52 ºC and 4.4´ 72 ºC was used followed by a 5 minute incubation at 72 ºC. The PCR products were pooled and precipitated with ethanol. The DNA was cut in a volume of 50 µl with 30 U of the endonucleases BamH I and Xba I respectively, and the reaction stopped by phenol extraction. In order to remove the cleaved ends, the DNA was incubated with Streptavidin-agarose (Sigma). In a microcentrifuge tube 100 µl of a 50 % (v/v) suspension of Streptavidin-agarose beads was washed 5 times with 1.5 ml of buffer (100 mM NaCl;. 1 mM EDTA; 10 mM Tris/HCl pH 8.0,). The beads were recovered by centrifugation in an Eppendorf microcentrifuge at 2,500 rpm and room temperature for 2 minutes. The DNA from the restriction step was added to the Streptavidin-agarose beads and incubated for 30 minutes at room temperature on a mixing platform. The beads were pelleted as before by centrifugation and the supernatant saved. The beads were then washed in 200 µl Milli-Q water and the supernatant pooled with the previous one. The pooled supernatants were extracted once with phenol/chloroform and once with chloroform. The success of the PCR, restriction cut and adsorption on Streptavidin Agarose was checked on a polyacrylamide gel.

The plasmid pME.A was cut with the restriction endonuclease Sal I and the linearized band purified on a preparative agarose gel. The band of interest was cut and extracted from the agarose using the Qiaex II kit (Qiagen). In order to minimize the contamination by uncut plasmid DNA the gel purification step was repeated. The purified DNA was dephosphorylated using Shrimp Alkaline Phosphatase. A ligation was set up containing 600 ng linearized plasmid DNA , 100 ng insert and 5 U T4 DNA Ligase in a total volume of 200 µl and incubated over night at 16 ºC. The DNA was precipitated with ethanol, resuspended in 50 µl Milli-Q water and incubated for 2 hours with the endonucleases Sal I and Xho I. The DNA was again precipitated with ethanol and resuspended in 10 µl Milli-Q water and 2 µl aliquots were electroporated into SURE2 bacteria. A total of 5

electroporations were done and the bacteria resuspended in 1 ml SOB medium each. Following a 30 minute incubation at 37 ºC, 2.5 ml each of the bacterial suspension was plated on a 20 cm x 20 cm LB agar dish containing Ampicillin and evenly spread. Small aliquots of the bacterial suspension were plated on smaller LB agar plates in order to determine the number of colonies.

5.14.2. *In vitro* selection of the plasmid library

For *in vitro* selection of plasmids, 90 µl of a 50 % (v/v) GSH agarose bead suspension were washed 3 times with 0.5 ml buffer A (100 mM NaCl; 10 mM $MgCl_2$; 0.1 % (w/v) NP-40; 10 mM Tris/HCl pH 8.0) by resuspending the beads in the buffer followed by centrifugation in an Eppendorf microcentrifuge at 2,500 rpm and room temperature for 2 minutes. The GST-Zα peptide was pre-adsorbed on the beads by combining 45 µl of the bead suspension with 2 µl GST-Zα (1.9 mg/ml) and 13 µl of buffer A. The sample was incubated for 30 minutes on a mixing platform at room temperature. Unbound peptide was removed by three washes with 0.5 ml buffer A. In a typical experiment, four different binding reactions were set up by combining either 20 ng of the plasmid library or 20 ng of plasmid pME.A with either GST-Zα adsorbed on GSH-Sepharose or just GSH-Sepharose. The samples were incubated for 30 minutes on a mixing platform at room temperature followed by three washes with 0.5 ml buffer A and two washes with 0.5 ml buffer B (200 mM NaCl; 10 mM $MgCl_2$; 0.1 % (w/v) NP-40; 10 mM Tris/HCl pH 8.0). Bound DNA was then eluted during a 20 minute incubation in buffer C (200 mM NaCl; 10 mM $MgCl_2$; 0.1 % (w/v) NP-40; 1 % (w/v) SDS;10 mM Tris/HCl pH 8.0) at 60 ºC. The eluate was extracted twice with phenol/chloroform to remove any traces of the beads and protein. After addition of 1 µg glycogen the eluates were precipitated with ethanol at –80 ºC for at least 2 hours. The DNA was resuspended in 10 µl Milli-Q water and five 1 µl aliquots were electroporated into bacteria and plated on two 20 cm x 20 cm LB agar dishes (Nunc). Following an overnight incubation at 37 ºC, the bacterial colonies were resuspended in 20 ml LB medium and aliquots frozen for long term storage. Of the remainder, 1.0 ml was used for a small scale plasmid preparation yielding enough DNA for the next round of selection. The concentration of the DNA was determined using a UV-photometer and the DNA diluted to 10 ng/µl.

5.15. *In vitro* selection of minicircles

5.15.1. Production of radioactively labeled minicircles

Minicircles were prepared as described in chapter 5.14.2, starting with DNA that had been obtained by two sequential PCR reactions, purified by Qiagen Qiaquick PCR purification spin columns, PEG precipitation and EcoR I digestion. The restricted DNA was purified again using Qiagen Qiaquick PCR purification spin columns. The concentration of the DNA solution obtained was measured using the Hoechst dye 33258. For radioactive labeling the DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP). In a 100 µl reaction, 1 µg DNA was incubated in the supplied buffer and 1 µl (1 U) CIAP were added. The sample was incubated for 1 h at 37 ºC. The enzyme was inactivated by addition of 1 µl 0.5 M EDTA pH 8.0 incubation at 70 ºC for 20 minutes. Afterwards the sample was extracted twice with phenol/chloroform/isoamylalcohol (24:24:1) and once with chloroform followed by precipitation with ethanol. For radioactive end labeling, the DNA was resuspended in 16.5 µl Milli-Q water, 2.5 µl supplied 10 x buffer and 1 µl (10 U) T4 Polynucleotide Kinase (MBI), followed by addition of 5 µl γ-[^{32}P]ATP (6,000 µCi/mmol; 10 µCi/µl). The sample was incubated for 30 minutes at 37 ºC. To assure complete phosphorylation 1 µl of 0.1 M ATP was added and the sample incubated for another 10 minutes at 37 ºC before the enzyme was inactivated by incubation for 20 minutes at 65 ºC. The end labeled DNA was purified from unincorporated nucleotides by addition of Milli-Q water to a final volume of 50 µl and centrifugation through a G50 Probequant spin column (Pharmacia). For ligation 200 µl of 5x ligation buffer (Life) 5 µl T4 DNA Ligase (Life; 1 U/µl)and Milli-Q water to a final volume of 1 ml and were added. The sample was incubated overnight at 15 ºC. The DNA was precipitated with ethanol.

5.15.2. Preparation of the minicircle library

The strategy used to prepare the minicircle library depends on two PCR reactions. In a first PCR reaction, a product of the desired length is amplified from a template plasmid. In order to allow for the circularization of the PCR product both primers contain a restriction enzyme site not present in the amplified region. Additionally, one of the

primers contains a stretch of randomized nucleotides and a sequence used as priming site in the second PCR reaction. In a second PCR reaction a primer complementary to the introduced priming site is used together with the other primer to amplify the PCR product further and ensure the generation of double stranded DNA.

Milli-Q water	78 µl
10 x buffer	10 µl
5 mM dNTP´s	4 µl
50 mM MgCl2	2 µl
50 µM pMEA273EcoRI primer	2 µl
50 µM N16-EcoRI-B primer	2 µl
0.1 µg/µl pME.JA cut with Pst I	1 µl
Taq DNA Polymerase 5 U/µl	1 µl

Table 5.6

The N16 library was generated by using the plasmid pME.JA as template. During the work it became apparent that linearization of the template plasmid DNA with the restriction enzyme Pst I greatly improved the efficiency of the PCR reaction. A first PCR reaction was done using the primers N16-EcoRI-B (CGG GAA TTC GGG AGA CAA GAA TAA ACG CTC AGN NNN NNN NNN NNN NNN GAA GGG AAT AAG GGC GAC ACG G) and pME.A273EcoRI (CGG GAA TTC GGG AGA CAA GAA TAA ACG CTC). During the chemical synthesis of the N16-EcoRI-B primer a fifth reagent channel containing all four Phosphoamidites mixed in the ratio A:C:G:T / 20:30:30:20 was used to synthesize the bases denoted with ´N´. The quality of the primer was checked by denaturing acrylamide gel electrophoresis and purified by gel electrophoresis if needed. The PCR conditions are given in Table 5.6 .A temperature program was used containing a 5´ incubation at 95 ºC followed by 15 cycles of 1 minute at 95 ºC, 1 minute at 45 ºC and 30 seconds at 72 ºC. The PCR product was purified using a Qiaquick PCR purification kit (Qiagen) and used as template in a second PCR reaction using the primers pMEA273EcoRI and EcoLib-1 (CGG GAA TTC GGG AGA CAA GAA TAA ACG CTC).

Milli-Q water	750 µl
10 x buffer	100 µl
dNTP´s	40 µl
50 mM MgCl2	20 µl
50 µM pMEA273EcoRI primer	20 µl
50 µM EcoLib-1 primer	20 µl
DNA from 1st PCR	40 µl
Taq DNA Polymerase 5 U/µl	10 µl

Table 5.7

A temperature program was used containing a 5 minute incubation at 95 ºC followed by 5 cycles of 1 minute at 95 ºC,

Milli-Q water	9.2 ml
1 M Tris/HCl pH 7.5	0.5 ml
1 M MgCl2	0.1 ml
0.1 M ATP	0.1 ml
1 M DTT	10 µl
10 mM Ethidium Bromide	5 µl
DNA	1 µg
T4 DNA Ligase 1 Weiss unit /µl	20 µl

Table 5.8

1 minute at 61 ºC and 30 seconds at 72 ºC. The PCR product was purified using 4 columns of the Qiaquick PCR purification kit (Qiagen), eluted in 4 x 50 µl TE pH 8.5 and precipitated with PEG. The DNA was resuspended in 100 µl Milli-Q water and incubated overnight with 50 U EcoR I. The next day the restriction enzyme was inactivated by incubating for 20 minutes at 70 ºC and the DNA purified using two columns of the Qiaquick PCR purification kit (Qiagen). The concentration of the DNA solution was determined using the fluorescent dye Hoechst 33258. The minicircles were then generated by ligation of 1 µg DNA in a total volume of 10 ml in the presence of 10 µM ethidium bromide and 20 U T4 DNA Ligase overnight at 16 ºC (Table 5.8). The next day, 20 µl of a 5 mg/ml solution of linear acrylamide was added to the ligation reaction and the DNA precipitated by the addition of 1 ml 3 M Sodium Acetate solution pH 5.2 and 25 ml ice cold ethanol. The sample was divided in two 30 ml Corex centrifuge tubes and the precipitated DNA collected by a centrifugation in a Sorvall HB-4 swing out rotor at 10,000 rpm (16,500 g) and 4 ºC for 30 minutes. The pellet was washed with 5 ml ice cold 70 % (v/v) ethanol and air dried. The DNA was resuspended in 2 x 100 µl TE pH 8.5 and stored frozen for the selection experiments.

5.15.3. *In vitro* selection of minicircles

The actual selection was performed by incubating the minicircles with GST-Zα immobilized on GSH-Sepharose. In all selection cycles a mock selection was performed in parallel, omitting the GST-Zα in order to assess the nonspecific binding of the minicircles to the agarose matrix and the surface of the Eppendorff microcentrifuge tubes. The GSH-Sepharose beads were prepared by resuspending 90 µl of a 50 % (v/v) bead slurry several times in 1 ml of buffer 1 (100 mM NaCl; 10 mM $MgCl_2$; 0.05 % (w/v) Tween 20; 10 mM Tris/HCl pH 8.0). When pipetting the beads it proved useful to cut off the tips of ordinary pipette tips. The beads were recovered by a centrifugation in an Eppendorf microcentrifuge at 2,500 rpm and room temperature for 2 minutes. After the final wash step the beads were resuspended in approximately the original volume and 15 µl to 30 µl bead suspension were used for the binding reactions. The beads were brought up to app. 50 µl with buffer 1 and the protein solution added. The samples were placed on a rocking platform and incubated for 30 minutes at room temperature.

Afterwards, the samples were washed with 3 times 1 ml buffer 1 in order to get rid of unbound protein. Equal volumes of DNA solution were then added to both Eppendorf tubes and the samples incubated as before for 30 minutes on a rocking platform. Unbound DNA was removed by washing 3 times with 1 ml buffer 1 and 2 times with buffer 2 (200 mM NaCl; 10 mM $MgCl_2$; 0.05 % (w/v) Tween 20; 10 mM Tris/HCl pH 8.0). Before the last wash the beads were transferred to a fresh microcentrifuge tube in order to eliminate DNA bound to the Eppendorf tube. Bound DNA was eluted by a 20 minute incubation in buffer 3 (100 mM NaCl; 10 mM $MgCl_2$; 0.05 % (w/v) Tween 20; 1 % (w/v) SDS 10 mM; Tris/HCl pH 8.0) at 60 ºC. The samples were then extracted twice with phenol/chloroform and once with chloroform.

The success of the selection step was analyzed by PCR amplification of the eluates. The number of PCR cycles necessary to detect a PCR product was used as a qualitative measure for the amount of DNA bound. Prior to PCR amplification a 10 µl aliquot of the eluated DNA was linearized with the restriction endonuclease EcoR I in a total volume of 20 µl. A PCR reaction mix was prepared as listed in Table 5.9. A PCR reaction was set up using 10 µl of the digest and 90 µl reaction mix. A thermocycler program of 5´ 95 ºC followed by 10 cycles of 1´ 95 ºC, 1´ 55 ºC and 1´ 72 ºC was used. After the 10th cycle, 5 µl aliquots were saved and the PCR continued for 20 more cycles with aliquots being saved after every second cycle. The aliquots were run on a 2 % (w/v) agarose gel and the difference in the number of cycles needed, until a PCR product was visible between the selected sample and the mock treated sample, used to determine the success of the selection

Milli-Q water	173 µl
10 x buffer	25 µl
5 mM dNTP´s	10 µl
50 mM $MgCl_2$	5 µl
50 µM EcoLib-1 primer	5 µl
50 µM pMEA273EcoRI primer	5 µl
Taq DNA Polymerase	2.5 µl

Table 5.9 PCR reaction mix

5.15.4. Amplification of selected minicircles

For amplification of the selected minicircles, 50 µl of the eluate was treated with EcoR I endonuclease over night. The next day the nuclease was inactivated by a 20 minute incubation at 65 ºC. A PCR reaction of 1 ml volume was set up as listed in Table 5.9 and split into 10 PCR tubes. The temperature program contained an incubation at 95 ºC for 5

Milli-Q water	730 µl
10 x buffer	100 µl
5 mM dNTP´s	40 µl
50 mM $MgCl_2$	20 µl
50 µM EcoLib-1 primer	20 µl
50 µM pMEA273EcoRI primer	20 µl
DNA	60 µl
Taq DNA Polymerase 5 U/µl	10 µl

Table 5.10

minutes followed by 20 cycles of 1 minute at 95 ºC, 1 minute at 61 ºC and 30 seconds at 72 ºC. The PCR reaction was pooled and purified using 2 Qiaquick PCR purification columns (Qiagen). Prior to the second PCR a test PCR was performed in a volume of 100 µl in order to test whether the number of PCR cycles generated too few or too much product. The second PCR reaction was then set up as listed in Table 5.10 in a final volume of 2 ml and split into 20 PCR tubes.

A temperature program containing a 5 minute incubation at 95 ºC followed by between 4 and 6 cycles of 1 minute at 95 ºC, 1 minute at 61 ºC and 30 seconds at 72 ºC. The PCR reaction was pooled and purified using 4 Qiaquick PCR purification columns (Qiagen) and eluted in 4 x 50 µl TE pH 8.5 . The eluate was pooled into 2 x 100 µl and PEG precipitated by the addition of 1 µl 1 M $MgCl_2$ and 35 µl of a 40 % (w/v) solution of PEG-4000. The samples were incubated for 2 hours at room temperature, followed by a centrifugation in an Eppendorf microcentrifuge at 13,000 rpm and room temperature for 30 minutes. The pellet was washed in 70 % (v/v) ethanol and air dried. The DNA pellets were resuspended in a total of 100 µl Milli-Q water and incubated over night with 50 U

Milli-Q water	1626 µl
10 x buffer	200 µl
5 mM dNTP´s	80 µl
50 mM $MgCl_2$	40 µl
1 mM EcoLib-1 primer	1 µl
1 mM pMEA273EcoRI primer	1 µl
DNA	40 µl
Taq DNA Polymerase 5 U/µl	20 µl

Table 5.11

EcoR I restriction endonuclease. The enzymatic reaction was stopped by incubating at 65 ºC for 20 minutes. The DNA was purified using two Qiaquick PCR purification columns (Qiagen) and eluted in 2 x 50 µl TE pH 8.5 . The concentration of the DNA was determined using the dye Hoechst 33258 . The amplified DNA was then ligated into minicircles as described for the generation of the initial library.

5.16. Commonly used buffers and solutions

ATP 0.1 M	A 0.1 M ATP solution was prepared by dissolving 55 mg ATP (Sigma A-3377 Type II from yeast) in Milli-Q water. The pH of the solution was adjusted by the stepwise addition of 1 N NaOH until a 1 µl aliquot spotted on indicator paper caused a coloration corresponding to pH 7.0.
EDTA 0.5 M, pH 8.0	186 g EDTA Disodium salt was added to 800 ml Milli-Q water. NaOH pellets were added under continuous stirring until the EDTA went into solution. The pH was adjusted to pH 8.0 with 10 N NaOH and the volume made up to 1 l with Milli-Q water.
LB medium	LB medium was prepared by dissolving 7.5 g Bacto-peptone, 7.5 g Yeast extract and 5 g NaCl in 1 l of demineralized water. The medium was autoclaved for 30 minutes at 120 ºC. Antibiotics were added, if required, after the medium had cooled down to below 50 ºC.
LB agar	LB agar was prepared the same way as LB medium except for the addition of 15 g agar.

Loading buffer for agarose gels	For a 10 x stock solution 0.4 g Ficoll (Type 400) were placed in a microcentrifuge tube and Milli-Q water added to a final volume of 1 ml. The Ficoll was dissolved by vortexing and heating to 60 ºC in a water bath. The dyes Bromphenol Blue and Xylene Cyanole FF were added as required.
PBS	PBS was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1 l Milli-Q water.
SOB	SOB medium was prepared by combining 20 g Bacto-Peptone, 5 g yeast extract, 0,5 g NaCl and 5 g $MgSO_4 \bullet 7H_2O$ in 1 ml twice distilled water. The solution was sterilized by autoclaving for 30 minutes at 120 ºC.
SOC	SOC medium was prepared from SOC medium by addition of a glucose solution sterilized by ultrafiltration to a final concentration of 20 mM.
Sodium acetate 3 M, pH 5.2	The sodium acetate was dissolved in 1/3 of the final volume of Milli-Q water. If the salt did not dissolve completely, glacial acetic acid was added. The pH was adjusted with glacial acetic acid to pH 5.2 and Milli-Q water added to the final volume.

20 x TAE (NaAc-buffer for agarose gels)	20 x TAE was prepared by adding 48.5 g Tris/Base, 4.1 g sodium acetate and 7.4 g disodium-EDTA. The pH was adjusted to 7.8 with glacial acetic acid and deionized water added to 1 l.
10 x TB	10 x TB was prepared by combining 107,8 g Tris-Base and 55,0 g boric acid. Deionized water was added to 1 l.
10 x TBE	10 x TBE was prepared by adding 538,9 g Tris-Base, 275,1 g boric acid and 37,2 g disodium-EDTA. Deionized water was added to 5 l.
TE	TE buffer consisted of 10 mM Tris/HCl adjusted to the required pH and 1 mM EDTA.

5.17. Bacterial strains

DH5α	F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E41 λ- *thi*-1 *gyr*A96 *rel*A1
SURE2	e14-(McrA$^-$) Δ(*mcr*CB-*hsd*SMR-*mrr*)171 *end*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 lac *rec*B *rec*J *sbc*C *Umu*(::Tn5 (Kan^r) *uvr*C) [F´ *pro*AB lacI^{q}ZΔ(M15 Tn10 (Tet^r)Amy Cam^r]
TOP10	F^-, *mcr*A d(*mrr-hsd*RMS-*mcr*BC) Δ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara-leu*) *gal*U *gal*K *rps*L(Str^r) *end*A1 *nup*G

5.18. Computer methods

The Z-score of DNA sequences was computed using the Interpret program of the MacMolly program package (Softgene, Berlin; Germany. The default settings were used (Scores : AT = 0; AC = 1; GT = 1; GC = 2 ; non alternating doublets = -1; maximal number of non-alternating doublets = 1) except for the minimal length of the alternating segment, which was set to 5, and the minimal total score, which was set to zero.